A Comparison of Bluetongue Virus and EHD Virus: Electronmicroscopy and Serology

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ABSTRACT

Bluetongue virus, BT_8, and the virus of epizootic hemorrhagic disease (EHD) of deer, NJ-55, were plaque purified and compared electronmicroscopically and serologically. The latter included a plaque reduction neutralization test, the agar gel precipitin test, and the complement fixation test. The viruses were indistinguishable morphologically, but antigenically different. A plaquing technique was described for EHD virus.

RÉSUMÉ

Les auteurs ont purifié par la méthode des plaques et comparé, au microscope électronique et sérologiquement, le virus du "bluetongue" BT_8 et le virus NJ-55 de l'hémorragie épizootique (EHD) du chevreuil. La comparaison séroligique comprenait une épreuve de neutralisation par plaque, un test de précipitation en milieu agar gélosé et une épreuve de fixation du complément.

Morphologiquement, les deux virus s'avérèrent identiques; ils se révélèrent cependant différents sérologiquement. On décrit une technique de plaquage pour le virus de l'hémorragie épizootique (EHD).

INTRODUCTION

Relatedness of bluetongue virus (BTV) and the virus of epizootic hemorrhagic disease (EHDV) of deer has been suggested on the basis of pathology, clinical syndrome, and epizootiological pattern in deer (7, 14). Such implications are of both fundamental and practical importance. The following report presents some morphological and serological comparisons.

METHODS AND MATERIALS

VIRUSES

The "standard" North American isolate of BTV, BT_8, was obtained from the bluetongue laboratory in Denver, Colorado[1], and subsequently cloned by three consecutive plaquings in L-929 cells. Virus stocks were produced in BHK_{21} cell cultures.[2]

EHDV, isolate NJ-55, was obtained from A. Wilhelm (16) who had obtained it from Shope (12). It was subsequently passed in BHK_{21} cell cultures.

Plaque purified BTV was neutralized by several reference antisera, both from our own and the Denver laboratories. EHDV was neutralized by our own reference antiserum and by EHDV antiserum kindly supplied by Drs. Tsai and Karstad, Ontario Veterinary College.[3] The latter sera had

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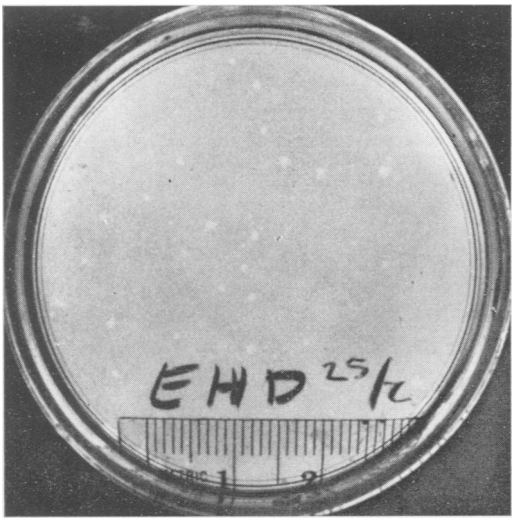

Fig. 1A. Epizootic hemorrhagic disease virus plaques.

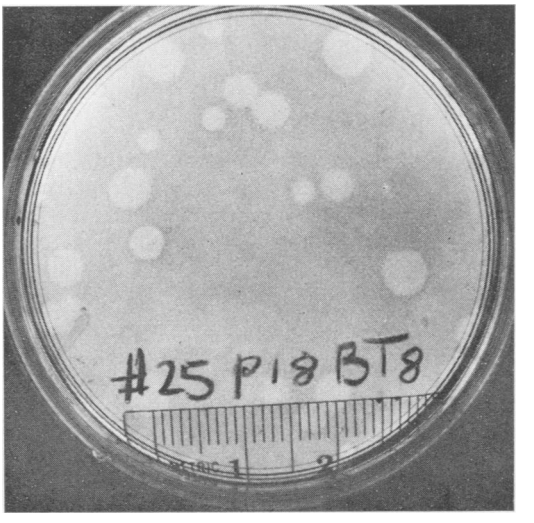

Fig. 1B. Bluetongue virus plaques.

been prepared against a Canadian isolate of EHDV ("Alberta Strain" (3)) in a deer. Antiserum prepared in deer against the cloned EHDV isolate reacted in the complement-fixation test with the EHDV antigen prepared by Dr. P. Boulanger, A.D. R.I., Hull, Que. (2). In addition to these methods of identifying the viruses, it is noted that the plaque types are quite distinct (Fig. 1, A and B). EHDV plaques were much smaller (pinpoint to 1 mm), irregularly shaped, and required seven days to develop. The BTV plaques were large (1-4 mm), round, and required only three days to develop. By these criteria, each virus stock was considered uncontaminated by the other.

Electron Microscopy

Negative Staining — Stocks of BTV and EHDV were removed from a -70°C freezer, thawed, clarified by low speed centrifugation, and used directly without further purification. The titers were approximately 10^7 to 10^8 plaque forming units (PFU) per 1 ml. Drops of the suspensions were mixed with 2% phosphotungstic acid on a glass slide, placed on carbon coated grids, and examined after drying with a Hitachi HV-11B electron microscope.

Thin Sections — Monolayer cultures of BHK_{21} cells in plastic petri dishes[4] were inoculated with BTV or EHDV at a multiplicity of infection of approximately 5 PFU per cell. Control cultures were inoculated with diluent. At various intervals after infection, cells were removed from the plastic with trypsin, washed in diluent (Hanks balanced salt solution with 10% fetal bovine serum added) and pelleted by low speed centrifugation. The pellets were immediately fixed with a solution containing 3.5% glutaraldehyde and 0.1 M cacodylate buffer, pH 7.2 at 4°C and then treated with 1% osmium tetroxide, dehydrated, and embedded in an araldite-epon mixture (9). Staining was done with uranyl acetate and lead citrate. Uninoculated control cultures were prepared at each of several sampling times post inoculation.

Antigenic Comparisons

Antisera — Antisera to both viruses were prepared in white-tailed deer. BTV antisera were taken from adult female deer infected six months previously with the vaccinal strain, BT_8, of BTV (13). EHDV antisera were prepared by subcutaneous inoculation of six month old deer with approximately 10^2 PFU of EHDV followed in two weeks by 10^7 PFU of the same virus. Sera were taken two weeks after the second inoculation. Sera were also obtained

[4]3002 Tissue Culture Dish, Falcon Plastics, 1950 Williams Dr., Oxnard Ca. 93030.

from non-inoculated animals that had been in contact with the inoculated animals.

Plaquing Technique

During the course of experiments with BTV, EHDV stocks were tested frequently in the plaquing technique used for the former to assure freedom from laboratory contamination with BTV. The plaquing procedure used was a modification of that described by Howell (5), the incubation time for plaque development being three or four days. When EHDV was used in this system and the inoculation time increased to seven days (made possible by the addition of a second overlay on the fourth day), small, irregular plaques appeared. These were determined to be caused by EHDV by the above criteria and EHDV was used in the plaque reduction neutralization (PRN) test as described for bluetongue virus (13).

The plaquing procedure was as follows: Monolayer cultures of L-929 cells were grown with minimum essential medium (MEM)[2] in plastic petri dishes[4]. After washing with diluent (Hanks balanced salt solution + 5% fetal bovine serum[2]), 0.1 ml of inoculum was put onto the monolayer. These were incubated in a humid atmosphere of 5% CO_2 at 37°C with frequent tilting to assure dispersal of the inoculum over the cells. At the end of this time the cultures were washed and 5 ml of overlay medium at 45°C applied. The latter consisted of equal volumes of double ionic strength (2X) MEM and 1.6% agar[5] in double distilled water. Diethylaminoethyl dextran (DEAE-dextran)[6] was added to a final concentration of 25 μg/ml and fetal bovine serum to a final concentration of 1% v/v. The overlay was allowed to solidify at room temperature, after which the plates were inverted and returned to the incubator. The BTV plates were stained after three days incubation and the EHDV plates were stained after seven days (a second 5 ml quantity of overlay having been applied at four days of incubation). The plates were stained by the addition of 1 ml of neutral red in growth medium to give a final concentration of 1 part neutral red to 10,000 parts overlay. The plaques were counted at four to 24 hours after staining for BTV and 24 hours after staining for EHDV.

[5]Ionagar No. 2, Colab Laboratories, Chicago Heights, Ill.
[6]DEAE-dextran, Pharmacia, Uppsala, Sweden.

Plaque Reduction Neutralization Test

The PRN test has been described (13). The ED50's were graphically calculated by the method of Russel *et al* (11), using several points on log probit paper.

Agar Gel Precipitin Test and Complement Fixation Test

Coded samples of EHDV hyperimmune sera were sent to Dr. M. M. Jochim at the Denver bluetongue laboratory, who tested them against BTV antigens in the agar gel precipitin (AGP) test described by Jochim and Chow (6). These and additional samples were coded and sent to Dr. E. Carbrey at the National Animal Disease Laboratory in Ames, Iowa, who tested them in the modified direct complement fixation (MD-CF) test as described by Boulanger *et al* (1).

RESULTS

Electronmicroscopy

Negative stained EHDV is shown in Fig. 2A, B, and C and BTV in Fig. 2 D, E, F.

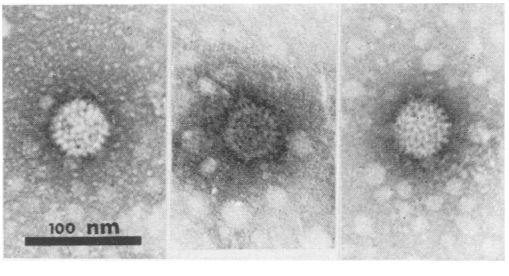

Fig. 2A, B and C. Epizootic hemorrhagic disease virus. x 152,000.

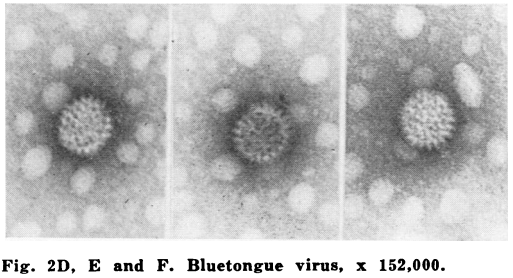

Fig. 2D, E and F. Bluetongue virus, x 152,000.

The size, shape and general capsid arrangement is strikingly similar for both viruses. Although it is impossible to estimate exact capsomere numbers by direct counting, this parameter and the size and shape of the capsomeres appears to be approximately the same for both viruses and consistent with Els and Verwoerd's description of 32 or 42 capsomeres for BTV (4). By direct measurement the diameter of the capsid was approximately 53 nanometers (nm) in both cases.

Thin sections taken at 24 hours post inoculation are shown in Fig. 3 and 4A, for EHDV and 4B, for BTV. Sections of the 40 hour samplings are seen in Fig. 5A and 5B for EHDV and BTV respectively.

In the 24 hour samples of EHDV, tubules and fibrils remarkably similar to those described by Lecatsas (8) for BTV were frequently seen. The tubules are demonstrated in association with viruses, their diameter corresponding roughly to that of the virions. The fibrils are apparent in the same picture (Fig. 3A).

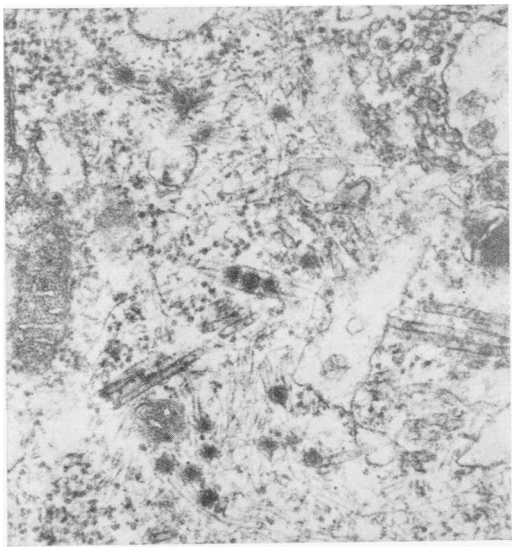

Fig. 3. EHDV in BHK_{21} cells, 24 hours post inoculation. x 38,000.

The 24 hour thin sections demonstrate a budding phenomenon observed rarely for both viruses. Viruses were seen to bud into cytoplasmic vacuoles or extracellular spaces, apparently retaining a closely applied host cell membrane component (Fig. 4A, B), the occurrence of such a process during the development of an ether resistant virus remains an enigma.

Another phenomenon common to both viruses was seen infrequently in the 40 hour section. Masses of free virions were seen in degenerating cells, all with some poorly defined material adjacent to the capsid (Fig. 5, A and B).

The marked similarity between the two viruses as described above is noteworthy.

No virions resembling EHDV or BTV were seen in control cultures. Structures resembling the "radial" virus observed by Ziegel *et al* (17) were seen in control and inoculated cells.

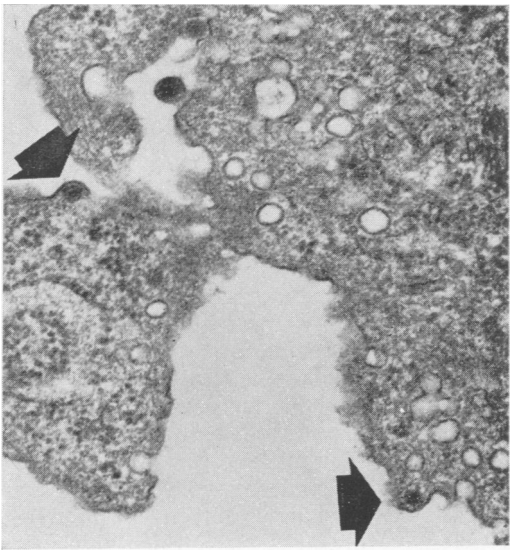

Fig. 4A. EHDV 24 hours post inoculation. x 46,000.

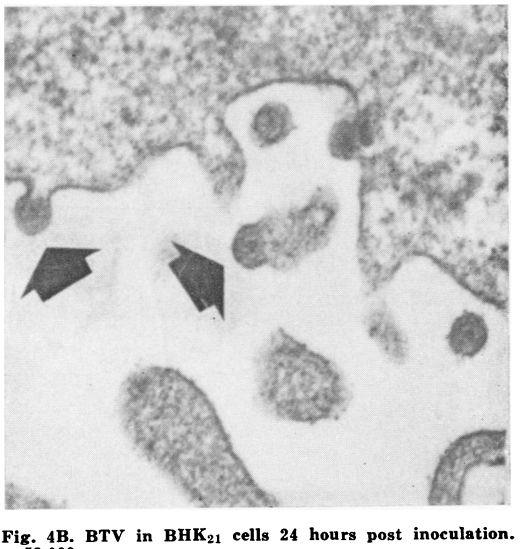

Fig. 4B. BTV in BHK_{21} cells 24 hours post inoculation. x 53,000.

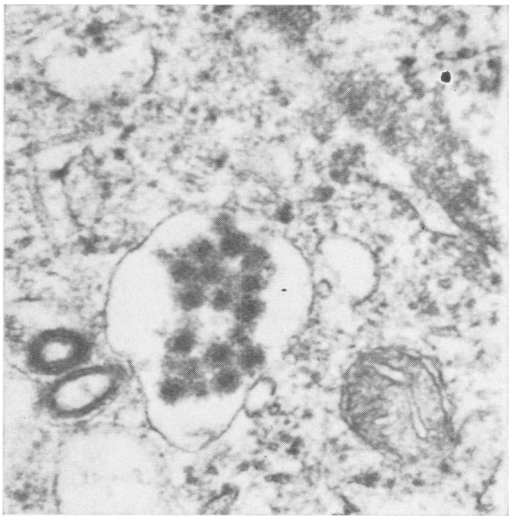

Fig. 5A. EHDV 40 hours post inoculation. x 73,000.

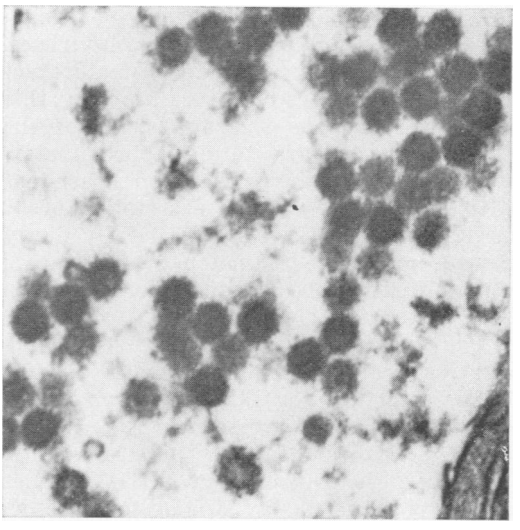

Fig. 5B. BTV 40 hours post inoculation. x 104,000.

ANTIGENIC RELATIONSHIPS

Plaque Reduction Neutralization Test — The results are summarized in Table I. In the face of homologous titers ranging from 1:3200 to 1:11,000 no cross reactions of comparable magnitude were seen. The low titers shown for the heterologous reactions and the preinoculation reactions were considered nonspecific inhibition as described previously for BTV (10). Such inhibition has been observed for most sera in our laboratory.

AGP and CF Tests

The CF and AGP tests failed to demonstrate any cross reactions (Table II) and thus were similar to the PRN test.

DISCUSSION

A plaquing technique has not been previously described for EHDV. The method described allows more precise quantitation of this virus and, as noted, supplies a sensitive method of antibody titration, the plaque reduction neutralization technique.

The marked physical similarities between

TABLE I. Plaque Reduction Neutralization Titers of Deer Infected with BTV or EHVD, Against Homologous and Heterologous Virus

Deer Number	Infecting Virus	BTV Titer		EHDV Titer	
		Pre-Inoculation	Post Inoculation	Pre-Inoculation	Post Inoculation
23-43	EHDV	1:4	1:5	1:5	1:11.000[a]
108-134	EHDV	1:2	1:4	1:26	1:3,800
35-37	None (in contact 23-43 and 108-134)	1:2	1:4	1:10	1:11
49	BTV	<1:5	1:3.200	—	<1:5
45	BTV	<1:5	1:6,900	—	<1:5
38	BTV	1:16	1:4.400	—	<1:5
95	BTV	1:7	1:10,000	—	<1:5
77	None (in contact 49, 45, 38, 95)	1:16	1:22	—	<1:5

[a]Post inoculation homologous titers are underlined. All others are considered nonspecific inhibition.

TABLE II. Results of Agar Gel Precipitin (AGP) and Complement Fixation (CF) Tests for BTV Antibody from Deer Infected with BTV or EHDV

Deer Number	Infecting Virus	AGP		CF	
		Pre-Inoculation	Post-Inoculation	Pre-Inoculation	Post-Inoculation
23-43	EHDV	Neg.	Neg.	Neg.	Neg.
108-134	EHDV	Neg.	Neg.	Neg.	Neg.
35-37	None (in contact 23-43 and 108-134)	—	—	Neg.	Neg.
49	BTV	—	—	—	Pos.[a]
92	BTV	—	—	—	Pos.
39	BTV	Neg.	Pos.	—	—
41	BTV	Neg.	Pos.	—	—
Pooled Sera	BTV	Neg.	Pos.	—	—

[a]All known positive BTV sera reactions underlined. Note that all others tested were negative.

EHDV and BTV is consistent with the hypothesis that these viruses belong to the same group. The serological tests suggest antigenically distinct viruses. The AGP and CF tests are said to detect a BTV group specific antigen (1, 6). The PRN test has been recognized as one of the most sensitive serological tests available and this is borne out in the magnitude of the homologous titers. It has also been observed in our laboratory that the different "strains" of BTV cross react to some extent in this test. This is thought to be a reflection of the sensitivity of the test. More powerful tools are available to detect small antigenic differences between viruses and these are presently being investigated. EHDV could be related antigenically to BTV strains other than BT_8. Until further work is done such as the characterization of the nucleic acid of EHDV and further examination of BTV and EHDV antigens, it might be useful to consider these viruses as antigenically distinct members of the same virus group.

Since the submission of this manuscript, work describing the electronmicroscopy of EHDV has been reported by Tsai and Karstad (15), many observations being similar to those reported here.

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